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Development of an Enzyme-Linked Immunosorbent Assay for the *Veratrum* Plant Teratogens: Cyclopamine and Jervine

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Veratrum californicum was responsible for large losses of sheep grazing high mountain ranges in central Idaho in the 1950s. *Veratrum* induces various birth defects including the cyclopic-type craniofacial defect (monkey-faced lambs) that is specifically induced in lambs after pregnant ewes grazed the plant on the 14th day of gestation. The steroidal alkaloids cyclopamine (**1**) and jervine (**2**) were isolated from *Veratrum* and shown to be primarily responsible for the malformations. Cyclopamine (**1**) and jervine (**2**) are potent teratogens that inhibit Sonic hedgehog (Shh) signaling during gastrulation-stage embryonic development, producing cyclopia and holoprosencephaly. Although losses to the sheep industry from *Veratrum* are now relatively infrequent, occasional incidents of toxicoses and craniofacial malformations are still reported in sheep and other species. However, the benefits to biomedical research using cyclopamine (**1**) as a tool to study human diseases have greatly expanded. A competitive inhibition enzyme-linked immunosorbent assay (ELISA) to detect and measure cyclopamine (**1**) and jervine (**2**) was developed using polyclonal antibodies produced in ewes. The limits of detection of the assay were 90.0 and 22.7 pg for cyclopamine (**1**) and jervine (**2**), respectively. This assay was used for the detection and measurement of cyclopamine (**1**) spiked into sheep blood. The simple extraction−ELISA methods developed in this study demonstrate the potential of using these techniques for the rapid screening of biological samples to detect the presence and concentration of cyclopamine (**1**) and jervine (**2**) and will be beneficial to pharmacological studies and livestock diagnostics.

KEYWORDS: ELISA; enzyme-linked immunoassay; cyclopamine; jervine; *Veratrum californicum*; alkaloids

INTRODUCTION

In the first half of the 20th century, sheep grazing the range plant *Veratrum californicum* in the high mountain ranges in central Idaho gave birth to a significant number of offspring with cyclopic and other craniofacial malformations (*1*). 11-Deoxojervine (**1**) and jervine (**2**) were later extracted from *V. californicum* and identified as the teratogens responsible for the malformations (*2*, *3*). Although Keeler gave 11-deoxojervine (**1**), the primary teratogen, the name cyclopamine (**1**), the alkaloid also induced malformations in the limbs and foregut at later gestational periods (*4*). More recently, molecular biologists reported that cyclopamine (**1**) exerted its biological response by inhibiting the Sonic hedgehog (Shh) signal transduction pathway (*5*, *6*). Members of the hedgehog family of secreted proteins serve as intercellular signals relayed between germ layers and function in a multitude of developmental processes that range from neural specification to bone morphogenesis (*7*). Several applications of cyclopamine (**1**) to inhibit mammalian Sonic hedgehog have correlated with the observations of terata in sheep. For example, the rapid, extensive expansion of the developing chick midbrain and forebrain have been retarded and overall head size was reduced following treatment with cyclopamine (*8*); treatment of regenerating fins in zebrafish with cyclopamine (**1**) leads to a reduction in fin outgrowth (*9*); and, mouse embryos exposed to jervine (**2**) partially phenocopied the lower jaw defects of Prx null mutants and further suffered loss of mandibular incisors (*10*).

A key research discovery that revealed the crucial role of the Sonic hedgehog gene in the developmental patterning of the mammalian forebrain used loss-of-function mutation at the Sonic hedgehog locus. Mouse embryos that lacked functional copies of Sonic hedgehog displayed severe holoprosencephaly that included cyclopia (*11*). Neutralization of Sonic hedgehog function may also be performed with the polyclonal antibody Ab 80 (*12*) or the anti-Sonic hedgehog monoclonal antibody 5E1 (*13*). More stringent inhibition of Sonic hedgehog signaling has been accomplished in many organ systems through the use of the plant steroidal alkaloid cyclopamine (**1**), which appears

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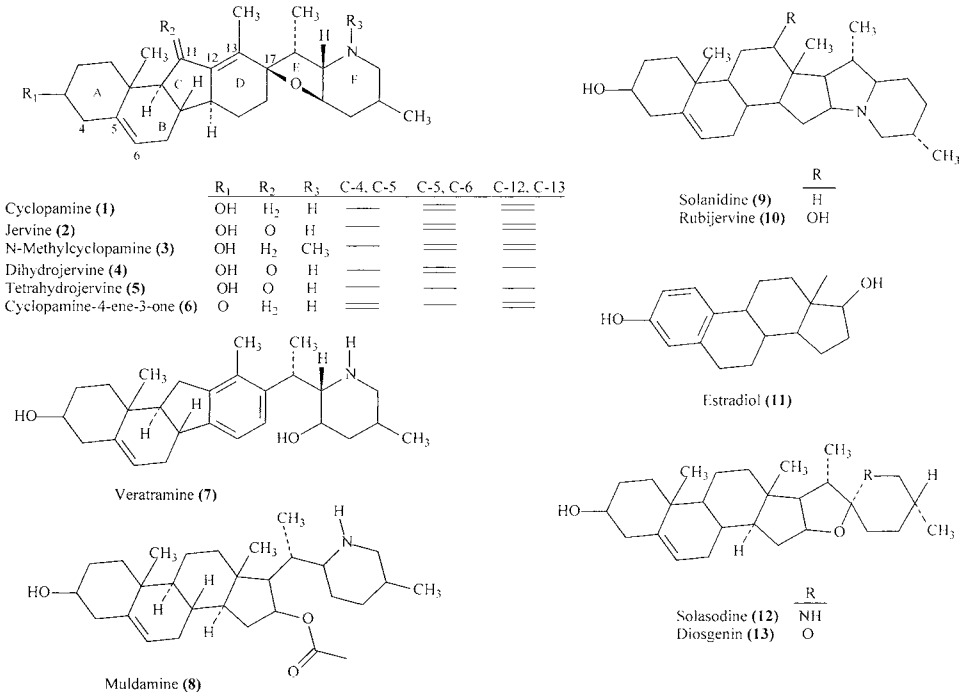

Figure 1. Chemical structures of cyclopamine (**1**) and other steroidal compounds tested for cross-reactivity with the antisera raised against the CYCLOSA-FET immunogen.

to act by altering the interaction between components of the Patched1−Smoothened receptor complex (*14*). As a result of its applicability to Sonic hedgehog inhibition, cyclopamine (**1**) is considered to be the primary prototype of a small molecule that reveals the logic and timing of vertebrate development (*15*). Thus, cyclopamine (**1**) offers a pharmacological tool to block Sonic hedgehog signaling that complements genetic and biochemical techniques (*16*).

Development of an enzyme-linked immunosorbent assay for cyclopamine (**1**) and jervine (**2**) may serve both agricultural and biomedical applications. Rapid, sensitive, and selective diagnostic agents are required to identify poisoned range animals and to determine the specific plants and conditions under which animals are likely to be poisoned. Tools to achieve these goals have been reported previously for toxins from *Delphinium* and *Senecio* (*17*, *18*). The analysis of the rate of clearance of cyclopamine (**1**) in mammalian blood could assist researchers in establishing the proper dosage protocol in biomedical experiments. Furthermore, the analysis of cyclopamine (**1**) present in a particular tissue or organ could be correlated with in situ hybridization data on genes related to the Sonic hedgehog pathway with regard to their expression or repression.

This investigation was designed to describe the development of a competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) to detect and measure cyclopamine (**1**) and jervine (**2**) in biological samples. ELISAs offer advantages of minimal sample preparation and the ability to run many samples simultaneously. Immunoassays are performed in aqueous media and are uniquely suited for the analysis of toxins in biological samples. We demonstrate the applicability of this assay for the detection and measurement of cyclopamine (**1**) in sheep blood.

MATERIALS AND METHODS

Chemicals. Fetuin from fetal calf serum, succinic anhydride, 3,3′,5,5′-tetramethylbenzidine, thimerosal, polyoxyethylene sorbitan monolaurate (Tween 20), and phosphate-buffered saline (PBS) tablets were obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Hydroxysuccinimide (NHS), 1,3-dicyclohexylcarbodiimide, ethylene glycol dimethyl ether, and silica 60 Å, 70−230 mesh, were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pyridine and dimethyl sulfoxide were purchased from Mallinckrodt Specialty Chemical Co. (Paris, KY). Chloroform and methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Bovine albumin (BSA) fraction V reagent grade was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). DEAE-dextran was purchased from Pharmacia Biotech (Uppsala, Sweden). Quil A saponin was obtained from Superfos Biosector a/s (Frydenlundsvej, Denmark). Montanide 888 was purchased from Seppic (Paris, France). Carnation nonfat dry milk was obtained from Nestle USA, Inc. (Solon, OH).

Cyclopamine (**1**), jervine (**2**), *N*-methylcyclopamine (**3**), dihydrojervine (**4**), tetrahydrojervine (**5**), cyclopamine-4-ene-3-one (**6**), veratramine (**7**), muldamine (**8**), solanidine (**9**), rubijervine (**10**), and solasidine (**12**) (**Figure 1**) were obtained from the sample collection of one of the authors (W.G.). Diosgenin (**13**) was purchased from Steraloids, Inc. (Newport, RI). β-Estradiol (**11**) was purchased from Sigma Chemical Co. (St. Louis, MO).

Alkaloid−protein conjugates were filtered and concentrated using an ultrafiltration cell from Amicon, Inc. (Beverly, MA) and 30000 MW cellulose ultrafiltration membranes purchased from Millipore Corp. (Bedford, MA). Enzyme-linked immunosorbent assays (ELISAs) were performed on 96-well NUNC F96 Maxisorp polystyrene microtiter plates purchased from VWR Scientific Products (Denver, CO). Microtiter plates were read with a Bio-Rad model 3550-UV microplate reader from Bio-Rad Laboratories (Hercules, CA) at 450 nm. Electrospray mass spectral data were acquired on a Finnigan LCQ mass spectrometer from Finnigan Corp. (San Jose, CA). Samples were loop injected into the electrospray source in a methanol/1% acetic acid (1:1) solution at a flow rate of 0.5 mL/min. Nonlinear curve fitting was performed using SigmaPlot software purchased from Jandel Scientific (Sausalito, CA).

Cyclopamine 3-Succinate Protein Conjugates. Cyclopamine (**1**) (20.0 mg, 4.87×10^{-2} mmol) and succinic anhydride (14.6 mg, 1.46×10^{-1} mmol) were added to a 1 mL reactivial containing a triangular stir bar. Pyridine (160 μL) was added and the mixture stirred continuously (18 h, 60 °C). The reaction was sampled and analyzed by electrospray mass spectrometry (ESMS). The mass spectrum indicated that ~80% of the cyclopamine ($MH^+ = 412$) had been reacted to form cyclopamine 3-succinate ($MH^+ = 512$). Cyclopamine 3-succinate (**14**) was isolated from the reaction mixture by column chro-

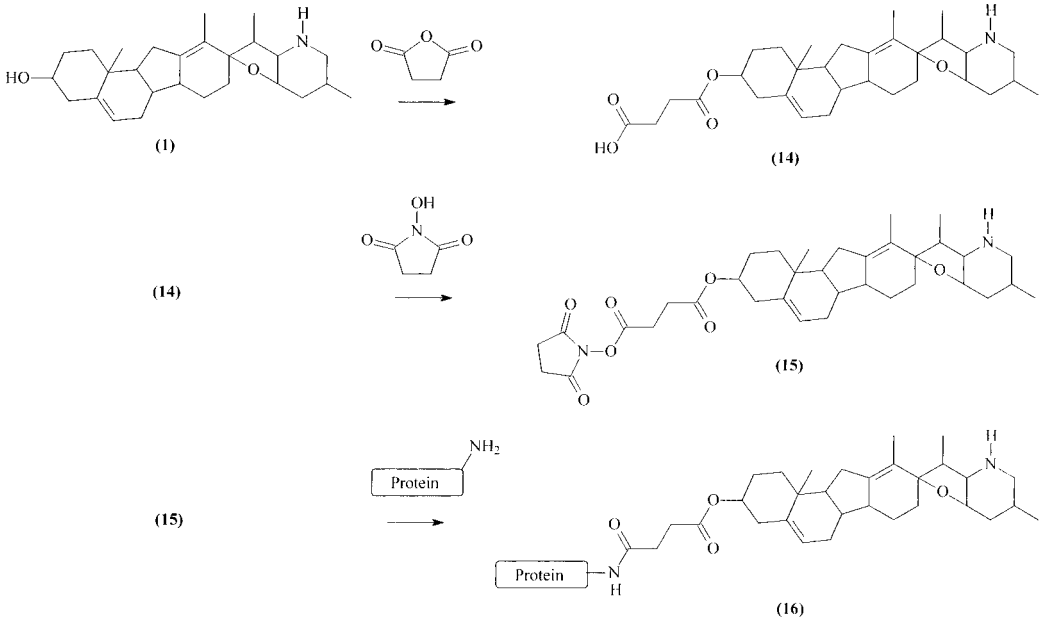

Figure 2. Synthesis of cyclopamine-3-succinate protein conjugates.

matography using silica as the stationary phase and chlorform/methanol/ammonium hydroxide (70:30:1, v/v/v) as the mobile phase. The product was confirmed by HRESIMS and ^{13}C NMR. Cyclopamine-3-succinate (**14**): HRESIMS (CH_3OH), 512.3386 (MH^+), $C_{31}H_{46}NO_5$ requires 512.3376; ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.1 (C═O), 176.2 (C═O). Other carbon resonances are similar to those for cyclopamine (**1**) (*19*).

Cyclopamine 3-succinate **(14)** (6.6 mg, 1.29×10^{-2} mmol), *N*-hydroxysuccinimide (2.6 mg, 2.26×10^{-2} mmol), and dicyclohexylcarbodiimide (4.6 mg, 2.23×10^{-2} mmol) were added to anhydrous chloroform (1 mL). The cyclopamine 3-succinate (**14**) and dicyclohexylcarbodiimide dissolved, whereas the NHS only partially dissolved. The reaction was stirred (16 h, room temperature), the reaction flask cooled with dry ice, and the solution filtered through a Büchner funnel and transferred to a 10 mL round-bottom flask. The chloroform was removed (in vacuo) at ambient temperature. The clear white residue was stored in a desiccator until conjugation with the protein.

The NHS-activated cyclopamine 3-succinate (**15**) was dissolved in dimethyl sulfoxide (250 μL). The dimethyl sulfoxide solution (200 μL) was added slowly to a continuously stirred solution of fetuin (24.5 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The remaining dimethyl sulfoxide solution (50 μL) was added slowly to a continuously stirred solution of BSA (24.2 mg) dissolved in 0.1 M $NaHCO_3$ (2 mL). The targeted conjugation ratios of these reactions were ~20:1 hapten to fetuin and ~7:1 hapten to BSA. These reactions were stirred (16 h, room temperature) and then filtered (0.45 μm syringe filter). The eluent was then filtered against a 30000 MW cutoff filter (five times) with deionized distilled water (50 mL). The product was then diluted to a concentration of ~1 mg/mL in deionized distilled water and stored (−120 °C) as 1 mL aliquots. These fetuin and BSA conjugates are designated CYCLO-SA-FET and CYCLO-SA-BSA, respectively, throughout this paper.

Immunizations. The primary injection solution was prepared by adding a 1 mg/mL hapten−fetuin conjugate in distilled water (6 mL) to a 1.25% DEAE-dextran, 0.125% Quil A, distilled water solution (9.6 mL) and emulsified with Montanide 888 (14.4 mL) for a total volume of ~30 mL. Two cross-bred ewes were initially injected subcutaneously with primary injection solution (2 mL) containing 0.4 mg of hapten−fetuin conjugate. Booster injections with half the concentration of hapten−fetuin conjugate (0.2 mg) in the above injection solution were given after three 6-week intervals. Blood samples were drawn immediately before the initial injection and 14 days after the second booster injection. Sera were stored at −95 °C.

ELISA Procedure. Cyclopamine−protein coating conjugates were dissolved in carbonate buffer (0.05 M, pH 9.6), and 100 μL was added to each well of the microtiter plate. The microtiter plates were incubated (2 h at room temperature and then 16 h at 4 °C). The plates were then inverted to remove excess coating solution and covered with an adhesive plate sealer and stored in a plastic bag (−20 °C) for up to 6 months. Before use, the plates were washed (three times) with saline−Tween buffer (0.15 M NaCl, 0.5% Tween 20) and blotted dry. Next, 150 μL of assay buffer (0.1 M Tris, pH 7.5, 0.1% Tween 20, 5% Carnation nonfat dry milk powder) was added, and the plates were incubated (1 h, room temperature). The assay buffer acted as a blocking buffer to reduce nonspecific binding of the antisera. The plates were washed (three times) and blotted dry. A 50 μL aliquot of samples or standards diluted in the assay buffer were added to the wells followed by 50 μL of antiserum diluted in the assay buffer. The plates were then incubated (2 h, room temperature). After the 2 h incubation, the plates were washed (four times), HRP conjugated donkey anti-sheep IgG (100 μL), diluted 1/10000 in assay buffer, was added to all wells, and the plates were incubated (1 h, room temperature). The plates were then washed (four times), and 100 μL of tetramethylbenzidine/H_2O_2 substrate (pH 5.5, 30 °C) (*20*) was added to each well. After 10 min, the reaction was stopped by the addition of 50 μL of 0.5 M H_2SO_4 to each well. The absorbances were measured at 450 nm (OD_{450}).

Plasma Preparation. Blood was drawn from a sheep into a vacuum tube containing sodium citrate to prevent clotting. Aliquots of blood were spiked with cyclopamine (**1**) to produce concentrations of 100, 500, 1000, and 6000 ppb (pg/μL), vortexed, and refrigerated for a minimum of 16 h. The samples were then centrifuged (2100 rpm, 30 min, room temperature). The plasma was removed from the samples with a pipet. Twenty microliters of the plasma was diluted into 980 μL of assay buffer and 50 μL applied to the microtiter plates.

RESULTS AND DISCUSSION

As a small molecule, cyclopamine (**1**) must be conjugated to a high molecular weight protein to elicit an immune response. The conjugation procedure included the reaction of the C-3 hydroxyl group of the alkaloid with succinic anhydride to form a hemisuccinate, followed by activation of the carboxylic acid moiety with NHS and reaction with the protein to form a four-atom-length succinate linker (**Figure 2**).

Antisera. Antibody titers for the CYCLO-SA-FET antisera were determined by titration of serial dilutions (1/500−1/256000) of sheep antisera against 250 ng/well of CYCLO-SA-BSA coating conjugate. Both sheep injected with conjugates produced antibodies. The sera from the two sheep that were

Table 1. ELISA Assay and Inhibition Data for Steroidal Alkaloids

	I_{50} (pg)	slope	LOD (I_{80}) (pg)	sensitivity
cyclopamine (**1**)	918	3.62	90.0	4.97
N-methylcyclopamine (**2**)	1.27×10^3	4.02	92.2	7.32
jervine (**3**)	776	1.62	22.7	7.11
dihydrojervine (**4**)	1.29×10^4	3.11	550	6.15
tetrahyrdrojervine (**5**)	8.83×10^4	5.72	6.21×10^3	15.5
cyclo-4-ene-3-one (**6**)	1.26×10^5	3.20	4.75×10^3	14.3
veratramine (**7**)	1.37×10^4	4.10	933	14.4
muldamine (**8**)	*a*	*a*	9.44×10^4	*a*
solanidine (**9**)	*a*	*a*	7.19×10^4	*a*
solasodine (**12**)	5.43×10^5	5.60	2.11×10^4	14.1

[a] The I_{50}, slope, and sensitivity are not reported if the maximum amount of compound tested was not ≥50% inhibition of the maximum absorbance.

injected with the same immunoconjugate were compared. The sera from the sheep that resulted in the highest titers were selected for further ELISA development.

A CI-ELISA assay was developed with the antisera raised against the CYCLO-SA-FET immunoconjugate and using the CYCLO-SA-BSA conjugate as a coating conjugate. The optimum dilutions for both the coating conjugate and antiserum were determined by checkerboard assays. The concentration of the coating conjugate and antiserum were selected on the basis of the combination at which the greatest difference in optical density was observed at 450 nm (OD_{450}) between the wells of no free cyclopamine (**1**) and the presence of cyclopamine (**1**) at levels of 0.1−10 ng/well, yet giving an optical density between 0.9 and 1.3 in the absence of free cyclopamine.

Cross-reactivity. Ten steroidal alkaloids (**Figure 1**) were selected for cross-reactivity studies: jervine (**2**), *N*-methylcyclopamine (**3**), dihydrojervine (**4**), tetrahydrojervine (**5**), cyclopamine-4-ene-3-one (**6**), veratramine (**7**), muldamine (**8**), solanidine (**9**), rubijervine (**10**), and solasodine (**12**). In addition, the steroids estradiol (**11**) and diosgenin (**13**) were tested for cross-reactivity. All steroidal compounds were tested for cross-reactivity over the range of $(1.95-1.024) \times 10^6$ pg/well. The data for each compound were fit to the four-parameter equation

$$y = (a - d)/[1 + (x/c)^b] + d \quad (1)$$

to yield inhibition curves. In this equation a and d are the upper and lower asymptotes, b is the slope of the linear portion of the curve, and c is the midpoint of the linear portion of the curve. **Table 1** reports the I_{50} values, the limit of detection (LOD) (I_{80}), the slope (b) of the curves, and the sensitivity of the assay for each compound. The constant c in eq 1 has been reported as I_{50} values for CI-ELISA assays (*21*). However, we chose to report the absolute I_{50} values as calculated from the equation of the curve. The LOD is conservatively defined in this study as the I_{80} value, as calculated from the equation of the curve. The sensitivity of the assay for each compound reported in **Table 1** is the product of the slope of the curve with the standard deviation of the experimental measurement closest to the I_{50} value of each compound.

Cyclopamine Assay. Checkerboard assays using the antiserum raised against the CYCLO-SA-FET immunogen with CYCLO-SA-BSA coating conjugate resulted in an optimum dilution of antisera of 1/12000 with the coating conjugate at 250 ng/well for a CI-ELISA assay. Inhibition curves for rubijervine (**10**), estradiol (**11**), and diosgenin (**13**) showed no cross-reactivity with the antibodies over the concentration range studied in this assay.

The inhibition curves in **Figure 3** and the I_{50} values reported in **Table 1** show cyclopamine (**1**), jervine (**2**), and *N*-methylcyclopamine (**3**) cross-react and are recognized by the antibodies raised against the CYCLO-SA-FET immunogen. **Figure 3** and **Table 1** also show that the antibodies in this assay have some affinity to dihydrojervine (**4**), tetrahydrojervine (**5**), cyclopamine-4-ene-3-one (**6**), and veratramine (**7**), whereas muldamine (**8**), solanidine (**9**), and solasodine (**12**) are recognized to an even lesser degree. From these data we conclude that the assay is specific for steroidal alkaloids possessing the structural features most similar to cyclopamine (**1**) and jervine (**2**). These compounds are C-*nor*-D-homosteroids with a fused furanopiperidine functional group attached spiro at the C-17 of the steroid. The addition of a ketone group at the C-11 position or the *N*-methyl alkylation of cyclopamine (**1**) as in jervine (**2**) and *N*-methylcyclopamine (**3**) does not impart enough structural dissimilarity to markedly reduce antibody recognition of these compounds compared to cyclopamine. However, saturation of jervine at the C-5/C-6 and C-12/C-13 positions, such as in dihydrojervine (**4**) and tetrahydrojervine (**5**), significantly reduces antibody recognition of these compounds. In addition, veratramine (**7**), which does not possess an ether-bridged furan ring, is not well recognized by the antibodies raised to the cyclopamine−protein conjugate. The LODs for cyclopamine (**1**), jervine (**2**), and *N*-methylcyclopamine (**3**) are 90.0, 22.7, and 92.2 pg, respectively.

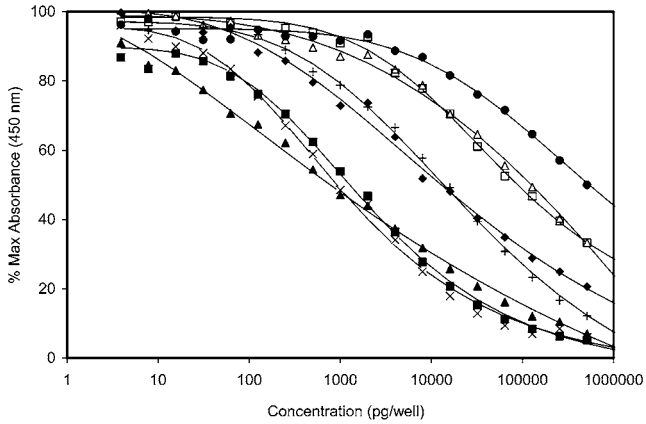

Figure 3. Inhibition curves for cyclopamine (×) $n = 9$, *N*-methylcyclopamine (■), jervine (▲), dihydrojervine (◆), tetrahydrojervine (□), veratramine (+), cyclo-4-ene-3-one (△), and solasodine (●). With the exception of cyclopamine all other points are the average of six replicates. RSDs for graphical data points ranged from 1.2 to 23%.

Determination of Cyclopamine (1) in Blood. Sheep blood was used to test the applicability of this assay for the detection and measurement of cyclopamine (**1**). Samples were quantified against a 10 point cyclopamine standard curve over the range of 15.6−8000 pg. The standard curve was determined by fitting the standards to eq 1. The r^2 for the standard curve was 0.9945. **Figure 4** shows the spike and recovery results for four different levels of cyclopamine (**1**) in blood. Average recoveries for 100, 500, 1000, and 6000 ppb cyclopamine (**1**) in blood ranged from 64 to 93%, whereas the relative standard deviations were 0.43−4.7%.

A CI-ELISA for cyclopamine (**1**) and jervine (**2**) was developed. The LODs of the assay were 90.0 and 22.7 pg for cyclopamine (**1**) and jervine (**2**), respectively. This assay was used for the detection and measurement of cyclopamine (**1**) in sheep blood. The simple extraction−ELISA methods described demonstrate the potential of using these techniques for the rapid screening of biological samples for the presence and levels of cyclopamine (**1**) and jervine (**2**). Although losses to the sheep industry from *Veratrum* are now relatively infrequent, occasional incidents of toxicoses and craniofacial malformations are still

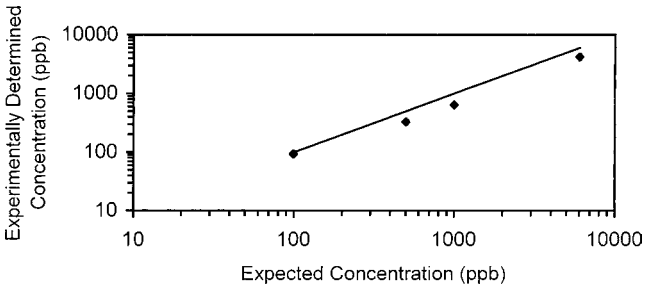

Figure 4. Correlation between spiked levels of cyclopamine and the level determined by the assay in sheep blood (◆). The line represents the theoretical correlation of cyclopamine levels.

reported in sheep and other species. In addition, biomedical interest in cyclopamine (**1**) as a tool to study human diseases continues to expand. This assay will be beneficial for identifying toxic plants, the diagnosis of poisoned animals, and pharmacological studies such as the study of cyclopamine (**1**) metabolism and clearance times in animal tissues.

ABBREVIATIONS USED

BSA, bovine serum albumin; CI-ELISA, competitive inhibition enzyme-linked immunosorbent assay; CID, collision-induced disassociation; CYCLO-SA-BSA, cyclopamine 3-succinate bovine serum albumin; ESMS, electrospray mass spectrometry; HRESIMS, high-resolution electrospray mass spectrometry; LOD, limit of detection; NHS, *N*-hydroxysuccinimide; I_{50}, mass of compound at which the absorbance at 450 nm is 50% of the maximum absorbance; I_{80}, mass of compound at which the absorbance at 450 nm is 80% of the maximum absorbance; NMR, nuclear magnetic resonance spectrometry; OD_{450}, optical density at 450 nm; PBS, phosphate-buffered saline solution (pH 7.4); PPRL, Poisonous Plant Research Laboratory; CYCLO-SA-FET, cyclopamine-3-succinate fetuin conjugate; CYCLO-SA-BSA, cyclopamine 3-succinate ovalbumin conjugate; Tween 20; polyoxethylene sorbitan monolaurate.

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